# 嘉南藥理科技大學專題研究計畫成果報告

以高效液相層析及可逆相管柱分離及純化肉桂酸之順反異購物

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Separation of the racemic mixtures of cinnamic acids by HPLC with a semi-preparative $C_{18}$ column

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摘要

在此研究中我們發展出利用高效液相層析來分離肉桂酸同分異構物的方法，分離順-反式肉桂酸的高效液相層析最佳條件為：移動相甲醇與去離子水 55:45(含 0.5 %醋酸)、移動相流速為 7.5 ml/min、樣品注射量則為 250μl。由此分離方法所純化出來的順-式肉桂酸的穩定性經測試後超過一年，此項結果建議利用高效液相層析法來分離肉桂酸同分異構物的方法比利用離子交換樹脂來分離肉桂酸同分異構物的方法更好且更準確。

關鍵詞：高效液相層析、同分異構物、肉桂酸、離子交換樹脂層析

Abstract

A HPLC method was developed to separate racemic mixtures of cinnamic acid in this study. The best conditions for the separation of trans-, and cis-cinnamic acids were: mobile phase MeOH and water : 55:45 (contains 0.5 % acetic acid, flow rate 7.5 ml/min, and injection volume 250 μl. The stability of the cis-isomer prepared by this study has been shown to be more than one year suggesting that the presenting HPLC preparation is a better and more precise method than the previous method of using ion exchange chromatography.

Key words: HPLC, racemic mixtures, cinnamic acid, ion exchange chromatography

## Introduction

Cinnamic acid is present in all kinds of plant derived foods, herbs, and medicines (Wolffram et al. 1994, Chen and Sheu 1995). Two forms of cinnamic acids, trans-cinnamic acid and cis-cinnamic acid, have been found to exist in the plant cells ($_{van}$ Overbeek 1951). However, the trans-cinnamic acid has been shown to be the predominate form in nature (>99%), since it is much more stable than the cis-isomer ($_{van}$ Overbeek 1951, Turner et al. 1993). Extensive studies have been reported for the metabolic pathways of trans-cinnamic acid in bacteria and eucaryotic cells (Floss 1979, Gross and Zenk 1969, Rosazza et al. 1993). The nutritional effects (Wolfrram et al. 1994), and biological functions of trans-cinnamic acid on bacteria, fungi, and animal cancer cells have been studied (Bitsch et

al 1984, Forti et al. 1996, Ekmekcioglu et al. 1998). cis-Cinnamic acid also plays an important role in the physiology of plant cells and fungi for the significant differences in the activities of bioactive enantiomers/diastereomers produced by or active against living microorganisms which are vitally important to pharmaceutical, flavor, and food industries (Hess et al. 1975, Aheldon 1993). However, due to the stability and purity of this unavailable cis-cinnamic acid, the mechanisms of cis-cinnamic acid on nutritional, toxicological, and metabolic pathway in eucaryotic cells are virtually unknown (Sun and Traxler 1998).

To date, the separation of cis-, and trans-cinnamic acids by ion-exchange chromatography method (based on pKa differences) was originally developed by Lindenfors in earily days (Lindenfors 1957). However, the elution of trans-cinnamic acid from that ion-exchange resin is a very slow process caused by strong attraction between the trans-, and cis-isomers of cinnamic acid. The strong partition effects between the trans-isomer of cinnamic acid and the packing resin also play a major factor for the poor separation of the racemic mixtures from the study by Lindenfors (1957). This is the likely reason that the cis-isomers of cinnamic acid obtained on that experiments were not stable (Lindenfors 1958). The cis-isomers of cinnamic acid obtained by that ion-exchange method probably contain a significant amount of trans-isomers. This may be the reason that the production of cis-cinnamic acid has not been commercialized so far.

In this paper, a simpler and much more precise method (based on difference in polarity) was present to separate cis-, and trans-cinnamic acids by using HPLC coupled with a semi-preparative $C_{18}$ column. The obtained pure cis-isomers were fully characterized by their melting points, UV spectra, FT-IR spectra, Mass spectra, and coupling constants (J). Moreover, the stabilities of these cis-isomers were also examined.

## Material and Methods

### Chemicals

trans-Cinnamic acid was obtained from Aldrich Chemical Co. (Milwaukee, WI) with the purities of 99.5%. All other chemicals were Baker Reagent (Phillipsburg, NJ) or Fisher Certified ACS (Fair Lawn, NJ). All solvents were obtained from Fisher Scientific Co. (Agawam, MA) and are HPLC or GC grade.

### Preparation of cis- and trans- cinnamic acid racemic mixture

To prepare cis- and trans-racemic mixture of cinnamic acid from the commercially available trans-compounds, 0.5 g of trans-cinnamic acid was dissolved in 10 ml acetone (GC grade) and placed in a

quartz tube under a photochemical chamber reactor and irradiated for 2 hr on a rotating plate surrounded by 16 ultraviolet lamps. Each lamp emits $1.65x10^{16}$/sec/$cm^3$ photons at 2537 $A^o$. After irradiation, the acetone was removed by sparging with $N_2$ until a dry residue was obtained. These residues were redissolved in 10 ml of methanol (HPLC grade) and filtered through a 0.45 μm membrane to remove any particulates and stored in brown bottles prior to HPLC separation.

**Separation of the cis- and trans- cinnamic acid racemic mixture by HPLC**

The Hiachi HPLC system (L7100 multisolvent delivery system) coupled with a L7455 photodiode array detector (PDA) and a semi-preparative $C_{18}$ reverse phase column (10.7 mm OD x 250 mm, Vercopak, Taiwan) were used to separate the cis- and trans-isomers. Methanol and deionized water mixtures were used as mobile phases for the separation of racemic mixture of cinnamic acid. In order to get a baseline separation, different mobile phase compositions and conditions were tested. The cis- and trans- isomers were detected by a Waters PDA detector set at 270 nm and scanned from 190-400 nm. The eluents of the cis-isomers were collected in 500 ml round bottom flasks with a cover of aluminum foil. The mobile phase was removed by a rotatory evaporator under a 20 psi vaccum at 65°C.

**The stability test of the cis-cinnamic acid obtained by this method**

The stability of the cis-isomer obtained after HPLC separation was monitored by the above HPLC system coupled with a Waters Nova-pak $C_{18}$ reverse phase analytical column (3.9 mm ID X 150 mm) and a mobile phase of 52 methanol : 48 deionized water (containing 0.5% acetic acid) was used. The flow rate was 1 ml/min and the injection volume was 2 μl. The chromatographs were extracted at 270 nm for quantification. The change in peak area was used as an index for the stability of the cis-isomer at the $1^{st}$ and $365^{th}$ day after preparation.

## Results:

**Separation of racemic mixture of cinnamic acids by HPLC with a semi-preparative $C_{18}$ column**

After 2 hr irradiation for trans-cinnamic acid in the photochemical chamber reactor, a ratio of 55 and 45 was determined to be the trans- and cis-form respectively, by using the HPLC with a semi-preparative $C_{18}$ column. In order to get a base line separation of racemic mixtures of cinnamic acids, different mobile phase composition, flow rate, and injection volume were tested. The mobile phase with a mixture of methanol (55 %) and deionized water mixture (45 %) under a flow rate of 7.5 ml/min was proven to be the best condition for the separation of

cis- and trans-cinnamic acids . After the trans- and cis-isomer was separated by the semi-preparative $C_{18}$ column, the cis-isomer was collected and used for the further stability tests.

## Discussions

The only difference for the chemical structure between the cis- and trans-cinnamic acid is the protons on #2 and #3 carbon for cis-cinnamic acid are out of the plane at the double bond (Turner et al. 1993). This difference makes these two isomers totally different in their chemical, physical, and biological properties (Lindenfor 1957, Hess et al. 1975, Sun and Traxler 1998).

The results of the stability tests of the cis-cinnamic acid indicate that the conversion of cis-isomer back to trans-isomer do not happen suggesting the cis-isomers prepared by this new method are fairly stable in a brown bottle at room temperature for at least 365 day. Althrough, the results from the earlier experiments (Lindenfors 1957, 1958) showed that the cis-isomers of cinnamic acid and chlorinated cinnamic acids prepared by the ion-exchange method were not stable and the reason causing this unstability was uncertain. Based on the studies conducted by Lindenfor (1957, 1958), the elution buffer with 0.4 M acetic acid and 0.3 M sodium chloride in 75% (v/v) ethanol solution was used for the ion-exchange chromatograpy. The strong attraction force between the racemic mixtures and partition effects of the resins, the high acidity and chloride ion concentration were probably insufficient to result in a good separation for these racemic mixtures of cinnamic acids. The cis-cinnamic acid obtained by that ion-exchange chromatography method, therefore, may contain significant amount of trans-isomer, acetic acid and chloride ion. Moreover, the cis-cinnamic acid obtained from the ion-exchange chromatography method resulted in sodium salt (white crystal) which are different from the transparent oil obtained by the developed HPLC method in this study. These differences are the likely reasons that the cis-isomer of cinnamic acid obtained in earlier experiments were not stable. Whereas, the cis-cinnamic acid obtained by the HPLC method developed in the study was a pure acid and containing neither acetic acid nor chloride ion. By comparing with the ion exchange chromatography method developed by Lindenfors (1957 and 1958), the present HPLC method may represent a faster, simpler and much more precise method for the separation of the racemic mixture of cis- and trans-cinnamic acids.

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